Bioactive lipids as key regulators in atherosclerosis

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1 General Introduction

Contents

1. Atherosclerosis

Atherosclerosis, a disease of medium- to large-sized arteries, is the primary cause of heart disease and stroke, and the major contributor to death in the world[1]. This chronic disease is already initiated in the second decade of life and is characterized by the accumulation of lipids and fibrous components in the arterial vascular wall[2]. Although clinical complications can be caused by plaques which display flow-limiting stenosis, the most severe clinical events are induced by plaque rupture, which exposes pro-thrombotic material entrapped in the plaque to the blood, initiating the coagulation cascade and causing luminal thrombus formation.
Atherosclerotic plaque formation occurs mainly at high risk areas such as branching points and bifurcations in the arterial tree[3,4]. The high vulnerability of these predilection sites to atherogenesis is attributable to hemodynamic factors, such as low shear stress, oscillatory flow and turbulent flow[5]. Atherosclerosis is a multifactorial disease in which lipids, inflammation, vascular potency and thrombosis all contribute to the development and final outcome of the disease. In the last decades a wide variety of risk factors have been identified for atherosclerosis, which can be divided in behavioral factors such as smoking, high fat diet, stress and physical inactivity, and genetic factors and disorders such as diabetes, dyslipidemia, hypertension, hyperhomocysteinemia and obesity, all acting in concert influencing the incidence of atherosclerosis[6-9]. Current therapies are mainly aimed at decreasing risk factors, such as lowering of plasma cholesterol by improvement of diet or the use of statins. Also hypertension can be beneficially influenced by lifestyle modification or medication. In addition, surgical intervention by e.g. bypass surgery, percutaneous transluminal coronary angioplasty (PTCA), stenting or atherectomy is frequently applied to restore impeded blood flow. The success rate of these interventions is often impaired by recurrence of lesions[10]. Despite the efficacy of these therapeutic measures, cardiovascular disease still continues to be the major cause of death in westernized societies, part of which is caused by patients who do not react to either lifestyle or pharmacological intervention. Therefore, the search for disease-targeted and tailor-made therapies against atherosclerosis is still a clinically highly relevant challenge.

2. Atherosclerotic Lesion Development

2.1 Lesion Initiation

The arterial wall normally consists of an endothelial layer covering a medial layer of smooth muscle cells flanked by internal and external elastic lamina. Outside the external elastic lamina, the artery is surrounded by adventitial tissue. The first step in lesion formation is endothelial barrier dysfunction by factors such as turbulent or oscillatory shear stress and certain risk factors (e.g. smoking, hypertension and elevated levels of atherogenic lipoproteins), which results in enhanced endothelial permeability and expression of adhesion molecules such as E- and P-selectin, which mediate monocytes “rolling” on top of the endothelium. Vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1 and some of the CC chemokine receptors (CCRs) enable the subsequent firm adherence of circulating leukocytes to the endothelium. These leukocytes, expressing among others P-selec-

tin glycoprotein ligand (PSGL)-1, very late antigen (VLA)-4 and CCR2[11-13], migrate through the endothelial layer into the subendothelial space (Figure 1A, 2A). The critical importance of these adhesion molecules was conclusively demonstrated in genetically altered mice. Deficiency or truncated forms of adhesion molecules such as P-selectin, E-selectin, VCAM-1, and CCR2, or deficiency for monocyte chemoattractant protein (MCP)-1, which is the ligand for CCR2, all show decreased plaque formation[14-18].

Once extravasated, monocytes will differentiate into tissue macrophages in the presence of a plethora of mediators such as macrophage colony stimulating factor (M-CSF) and tumor necrosis factor (TNF)-α, derived from residing tissue macrophages[19,20]. In addition, monocyte differentiation can be induced by growth factors like transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF)-1.

But not only monocytes are instrumental in the initiation of atherosclerosis. Besides influx of monocytes, the initiation of atherosclerosis is characterized by a concomitant lipid influx and a disturbed lipid metabolism, which are also crucial contributors in this process. Due to the increased permeability of the endothelial barrier and thus of the vessel wall, lipoproteins and especially low-density lipoprotein (LDL)-cholesterol can penetrate and stick to proteoglycans in the vessel wall. LDL-particles will, by virtue of oxidative stress in the subendothelial tissue, be modified into minimally-modified LDL (mmLDL) or mildly-oxidized LDL (moxLDL), become more extensively oxidized (oxLDL) and be taken up via scavenger receptors (CD36, CD68, CXC chemokine ligand 16, lectin type oxLDL receptors 1, scavenger receptor A and BI and macrophage receptor with collagenous structure) by subendothelially accumulated tissue macrophages[21-23], which in turn develop into foam cells. Altogether, this will subsequently lead to early lesion development (Figure 1B).

As the lesion progresses, inflammatory processes initiated by tissue macrophages will, under the influence of chemotactic molecules, attract other inflammatory cells such as T cells, which can produce interferon (IFN)-γ, TNF-α and pro-inflammatory interleukins (e.g. interleukin [IL]-1 and 2), thereby promoting lesion progression. Additionally, vascular smooth muscle cells (VSMCs) begin to migrate towards the luminal side of the lesion under the influence of PDGF, fibroblast growth factor (FGF) and TGF-β[24].

2.2 Lesion Progression

The early lesions, fatty streaks, are clinically asymptomatic, but can progress to more intermediate lesions characterized by the accumulation of extracellular lipid-rich debris due to either apoptosis/necrosis of intimal lipid-laden macrophages or disassembly of infiltrated lipoprotein particles under a layer of migrated VSMCs. These plaques are considered as true atherosclerotic or pre-atheroma plaques[25-27], and further progress to advanced and more complex lesions. The intimal lipid deposits will expand into large cell-free lipid pools containing a substantial amount of cholesterol crystals. At this point in development the lesion is referred to as an atheroma and in the central atheroma hypoxia will occur due to large distances to the vascular supply and thus to necessary nutrients. Therefore, this central atheroma has to be nurtured by microvessels sprouted from the vasa vasorum, which is a network of small arterioles, capillaries and venules that supply the perivascular tissue of

large blood vessels of essential materials. Formation and functionality of these vasa vasora-derived neovessels are regulated via an organized system of sympathetic and hormonal stimuli and they represent a permanent communication route between circulation and the central atheroma, allowing the influx of detrimental agents and hematopoietic subsets, such as monocytes and erythrocytes[28].

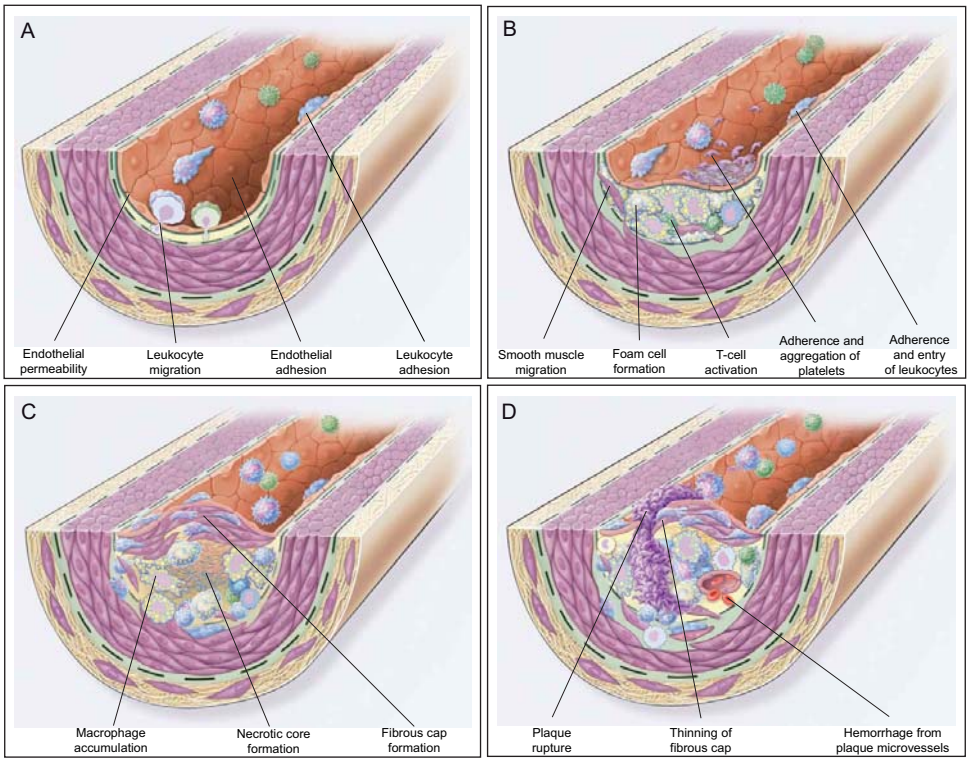

Figure 1. (A) Endothelial dysfunction in atherosclerosis. (B) Fatty-streak formation. (C) Formation of an advanced, complicated lesion. (D) Unstable fibrous plaques. (adapted from Ross R. *N Eng J Med.* 1999)[19].

At later stages more VSMCs will migrate to the luminal side of the lesion and proliferate to accumulate subendothelially and produce extracellular matrix material like collagen and proteoglycans, forming a fibrous cap, which covers the lipid core[24] (Figure 1C). Plaques at this stage of development are called fibro-atheromas[29] and are freely exposed to blood flow forces. They will become biomechanically vulnerably after fibrous cap erosion and most plaque ruptures take place in this lesion type[30]. The final stage of lesion progression represents ruptured or eroded lesions with intramural or luminal thrombi, or lesions containing hemorrhage (Figure 1D). This end stage of disease is described as atherothrombosis, defined as the process in which atherosclerotic lesions develop a thrombus, and is characterized by a ruptured atherosclerotic lesion containing superimposed thrombi. In fact, it is the major cause of the acute coronary syndromes (e.g. myocardial infarction, stroke, transient ischemic attack [TIA] or peripheral artery diseases) and death[31].

3. Unstable Lesions

Plaques that have progressed to the thin-cap fibro-atheroma stage or further are considered “unstable”. These plaques are responsible for the majority of clinical manifestations as stroke and myocardial infarction. When the balance between the size and consistency of the necrotic core and the strength of the fibrous cap is disturbed, the fibrous cap may rupture leading to direct contact of the highly thrombogenic content of the lipid core with the circulation and activation of the coagulation system. Several factors are thought to reduce the stability of atherosclerotic lesions, including matrix degradation, fibrous cap degradation and lipid core enlargement. The small microvessels that contribute to plaque neovascularization are often dysfunctional and do not contain a pericyte sheet, which makes them vulnerable for intraplaque hemorrhage which destabilizes the lesion[30]. Additionally, inflammatory cells present in the plaque can influence its instability. IFN-γ produced by T helper 1 cells can cause instability by its inhibitory effect on VSMC proliferation and collagen production[32,33]. Furthermore, macrophages can reduce plaque stability via the production of matrix-degrading proteases such as matrix metalloproteinase (MMP)-1, MMP-8, MMP-9 and MMP-13[34-37] and cathepsins[38]. Also the presence of mast cells in the adventitia deteriorates lesion progression and increased mast cell activation increases the vulnerability of lesions[39,40].

4. Research Models

Preclinical research on atherosclerosis largely depends on representative *in vitro* and *in vivo* models. Specific responses of individual cell types to atherogenic stimuli are best studied in *in vitro* models. As this lacks the complexity and cross interactions with multiple cell types it does not come near the complexity of the human atheroma. Animal models of atherosclerosis on the other hand may provide information of the net effect in a complex disorder such as atherosclerosis and may thus be particularly useful for the preclinical screening of therapeutic strategies. Results obtained in different animal models can, however, not always be extrapolated to the human situation as not all processes are regulated in the exact same way in different species. Numerous species have been used to elucidate the mechanisms of atherosclerotic lesion development, such as non-human primates[29], swine[41], rabbits[42,43], rats and transgenic mice.

The mouse has emerged as the model of choice in atherosclerosis research because of the advantages that they are small, relatively cheap and currently several transgenic and knockout mice are available to study the role of single genes in this disease. Conventional wild-type mice are not suitable for studies on atherosclerotic lesion development because of their high resistance to atherogenic stimuli. Even lesion-prone C57Bl/6 mice only develop small fatty streak-like lesions in the aorta when fed a rather unphysiological high cholesterol, cholate containing diet[44]. Hyperlipidemic mice that are prone to lesion development are the apolipoprotein E deficient ($ApoE^{-/-}$)[45,46], the ApoE*3-Leiden transgenic[47] and the LDL receptor deficient ($LDLr^{-/-}$) mouse[48]. While the latter two develop atherosclerosis when fed a high cholesterol diet, the $ApoE^{-/-}$ mouse already suffers from hypercholesterolemia on chow diet and

spontaneously develop large, complex atherosclerotic plaques[49]. These lesions are characterized by foam cell formation, a smooth muscle cell cap, lipid accumulation, high collagen content and the presence of a necrotic core. Similar to humans with familial hypercholesterolemia having defective LDL receptors, LDL receptor deficient mice have elevated levels of total cholesterol upon feeding of a high cholesterol diet and develop macrophage-rich lesions. Compared to ApoE$^{-/-}$ mice, atherosclerotic lesions in LDLr$^{-/-}$ mice develop more slowly and are less severe. The ApoE*3-Leiden transgenic mouse has been developed as a model for familial dysbetalipoproteinemia[47]. These mice express a dominant dysfunctional lipoprotein E*3-Leiden and these mice exhibit high levels of cholesterol and high triglyceride levels, mainly in very-low-density lipoprotein (VLDL) and LDL, which results in initial and advanced atherosclerotic lesions in the sinus valves and the carotid arteries upon cholesterol feeding[47].
As lesion development in these atherosclerosis-prone mice still can take months to develop and sites of lesion development are difficult to reach for experimental manipulation, strategies have been elaborated to speed up atherogenesis. Atherosclerosis was considerably accelerated after placement of a silastic collar or a cylinder with a tapered lumen perivascularly at the carotid arteries of hypercholesterolemic mice[50,51]. Lesion formation in these models was shown to be completely lipid and flow dependent. When studying atherothrombosis in mice, we have to deal with the attendant fact that in mice true and spontaneous plaque rupture and subsequent thrombus formation has hardly ever been observed[52]. Johnson *et al.* have thoroughly investigated the brachiocephalic artery of ApoE$^{-/-}$ mice for indications of plaque rupture[53]. While they did observe intraplaque hemorrhage and buried caps that were tentatively claimed to represent healed cap ruptures, no actual thrombotic occlusions but fibrin deposits were observed. Thus, the relevance of this model for plaque rupture research has been disputed[54-56]. However, intraplaque hemorrhage is a phenomenon which is more often observed in mouse models as compared to plaque rupture (either spontaneous or after intervention)[57,58] or thrombus formation and as described previously, intraplaque hemorrhage is deemed to be associated with plaque destabilization[59,60].

As different transgenic and knockout mouse models are developed, more and more research questions are addressed on involvement of specific proteins in different stages of atherosclerotic lesion development. However, to investigate the role of these proteins in atherosclerosis most of these animals need to be backcrossed to mice with an atherosclerotic-prone background as strains can differ quite considerably in their susceptibility to atherosclerosis[61]. As this is often very laborious due to the necessity of backcrossing 9 generations into a specific background strain and sometimes only the effects of leukocyte expression of these proteins are of relevance, a total-body transgenic or knockout is unsuitable. It also happens that transgenics or knockouts are embryonically lethal or die 2-3 weeks after gestation, which also hinders investigation of the role of these proteins in atherosclerosis development. Since 1995 it has been possible to partly circumvent these issues and perform bone marrow transplantations in mouse models for atherosclerosis[62-65]. Lethal irradiation of the animals will destroy their endogenous bone marrow and, by intravenously injecting donor bone marrow cells or fetal liver cells from transgenic or

knockout animals (on a corresponding genetic background), repopulation will take place of bone marrow expressing or lacking the gene of interest. This gives rise to a new area of leukocyte targeted research[64-67], in which the contribution of hematopoietic expression of these genes can be investigated. In addition, this technique gives the possibility to differentiate between the contribution of hematopoietic versus non-hematopoietic gene expression.

5. Plaque Inflammation

5.1 Macrophages

The atherosclerotic plaque contains different cell types such as endothelial cells, vascular smooth muscle cells, macrophages and T lymphocytes, which all can express inflammatory mediators in response to injury. Next to vascular smooth muscle cells, macrophages are the most abundant cell type within the lesion[19]. They are part of the innate immune system responsible for the first line of defense against pathogens. The uptake of oxLDL by macrophages via the scavenger receptors not only leads to cell activation but also results in the formation of foam cells (Figure 2B)[21,23]. Another pathway for macrophage activation proceeds through Toll-like receptors (TLRs, Figure 2B)[11]. Activation of these receptors by e.g. bacterial toxins such as lipopolysaccharide (LPS), stress proteins such as heat shock protein (HSP)60, but also by oxLDL, will trigger the production and secretion of pro-inflammatory cytokines such as TNF-α, which is considered pro-atherogenic. Important as antigen-presenting cells (APCs), macrophages will process the ingested oxLDL. Epitopes derived from oxLDL will be presented on major histocompatibility complex (MHC) class II molecules and can, via T cell receptor (TCR), activate antigen specific $CD4^+$ T cells to induce an epitope-specific humoral or cellular immune response (Figure 2C).

5.2 T Cells

A possible role of T cells within human atherosclerotic plaques has been described already in 1986[68]. It has now become clear that the majority of T cells present in or nearby the atherosclerotic lesion are activated $CD4^+$ T cells and that activated T-cell numbers increase with the severity of coronary syndrome[69-71]. Their importance in atherogenesis has been demonstrated by multiple studies in $ApoE^{-/-}$ or $LDLr^{-/-}$ mice. Deficiency in $CD4^+$ T cells and thus a deficiency in adaptive immunity leads to reduced atherosclerosis, while transfer of $CD4^+$ T cells accelerates atherosclerosis in immune deficient scid/scid mice[72-75]. Depletion of $CD4^+$ T cells via antibody administration or CD4 deficiency reduced fatty streak formation in C57Bl/6 mice[76]. $CD8^+$ T cells have also been detected within the human atherosclerotic lesions[71], but contrasting data exist on their role in atherosclerosis, most likely depending on which subset of $CD8^+$ T cells are targeted (memory $CD8^+$ T cells or cytotoxic $CD8^+$ T cells). Absence of the total $CD8^+$ T-cell population ($ApoE^{-/-}CD8^{-/-}$ mice) has no effect on lesion formation[75], while another study demonstrated an acceleration of atherogenesis due to $CD8^+$ T-cell activation[77].

$CD4^+$ T cells can be subdivided in several subclasses such as T helper (Th) cells and regulatory T cells (Treg). The T helper cells can be further categorized in Th1 and Th2 cells based upon their secretion pattern of cytokines, which are immune

modulators that mediate and control inflammatory responses. Th1 cells produce pro-inflammatory cytokines such as IL-1, IL-2, IFN-γ, IL-12, IL-18, and TNF-α, and are regarded pro-atherogenic, while the Th2 subpopulation, which produce cytokines such as IL-4, IL-5, IL-10, IL-13 and TGF-β, is considered mainly anti-atherogenic. As levels of IL-2 and IFN-γ are elevated in atherosclerotic lesions[71], most of the $CD4^+$ T cells within the atherosclerotic lesion are of the Th1 type. These Th1 cells can, by secreting cytokines and by direct binding to macrophages, stimulate these macrophages to produce more pro-inflammatory cytokines (Figure 2D)[11,71].

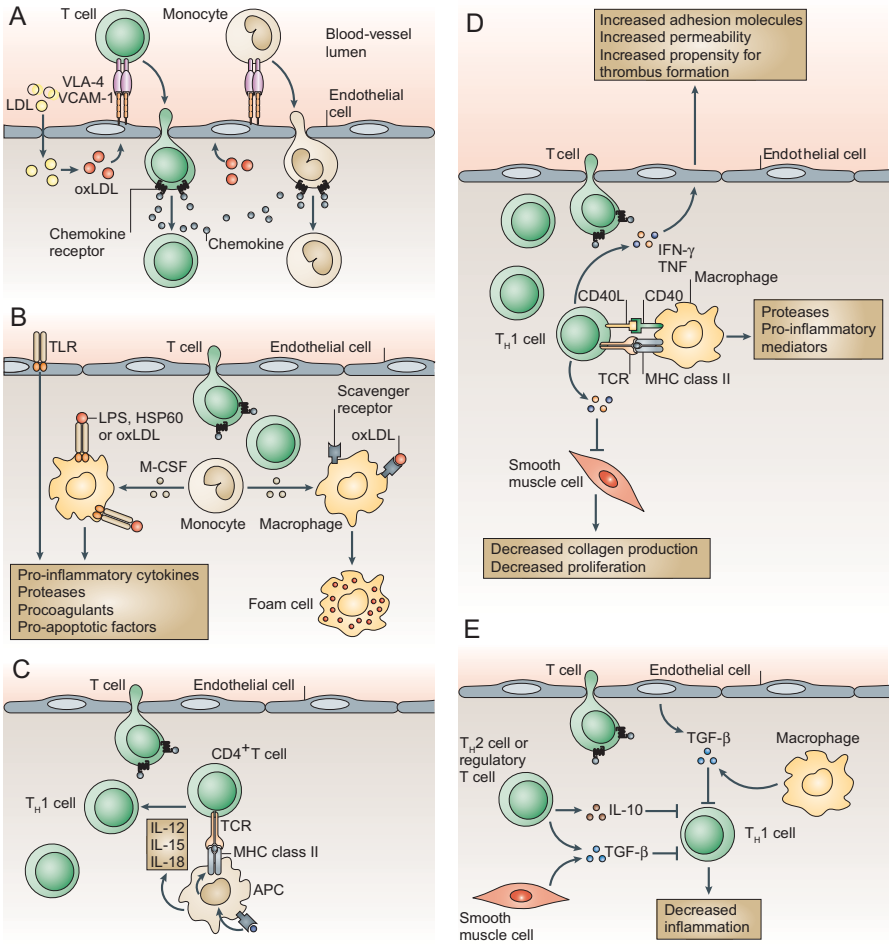

Figure 2. (A) Diffusion of low-density lipoprotein (LDL) and migration of monocytes and T cells into the arterial tissue. (B) Macrophage activation and foam cell formation. (C) T cell activation by antigen-presenting cells (APCs). (D) Th1 cells produce cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF), and express CD40 ligand (CD40L), by which they can activate endothelial cells and macrophages. (E) Regulatory T cells and macrophages can produce anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β, which might attenuate plaque inflammation. VLA-4, very late antigen-4; VCAM-1, Vascular cell adhesion molecule-1; oxLDL, oxidized LDL; TLR, Toll-like receptor; LPS, lipopolysaccharide; HSP60, heat shock protein 60; M-CSF, macrophage colony stimulating factor; MHC, major histocompatibility complex; TCR, T-cell receptor. (Adapted from Hansson GK, Libby P. *Nat Rev Immunol.* 2006)[11].

The pro-inflammatory and atherogenic potential of the Th1 cytokines has been demonstrated in multiple animal studies on IL-1 and its natural inhibitor IL-1 receptor antagonist[78-87], IL-2[88], IFN-γ[89-93], IL-12[94,95], IL-18[96,97] and TNF-α[98], of which the latter is also produced by macrophages and other cell types.
Th2 cytokines are considered mostly atheroprotective (Figure 2E). This notion is supported by studies performed on IL-5[99], IL-10[100-105] and TGF-β[106-110]. Next to the dampening effect on atherogenic T cell responses and inhibition of leukocyte recruitment, TGF-β can also affect plaque stability as it has the capacity to induce collagen synthesis and tissue inhibitors of MMPs and to inhibit foam cell formation. Conversely, data on IL-4, a prototype of a Th2 cytokine, are inconclusive and appear dependent on the stage of atherosclerosis, IL-4 being anti-atherogenic at early and pro-atherogenic at advanced stages of atherosclerosis[76,111-114]. These divergent findings, under different experimental conditions reflect the functional complexity of IL-4. Therefore, defining the role of Th2 cells in atherosclerosis needs further study.
In atherosclerosis, Th function is thought to be biased towards a Th1 type response, which is supported by the fact that C57Bl/6 mice, prone to a Th1 type immune response, develop fatty streaks on high cholesterol diet, while BALB/c mice, prone to Th2 immune responses, are protected against atherosclerosis[44,76]. Although tempting this Th1/Th2 theory has its limitations as some Th2 cytokines may promote progression of atherosclerotic lesions at certain stages. A new, distinct subset of T cells has been discovered, the Tregs, which can suppress both the Th1 and Th2 pathogenic immune responses against foreign or self-antigens, and in this way control T cell homeostasis[115,116]. Stimulation of Treg activity has been demonstrated to attenuate atherosclerotic lesion development[117,118]. The mode of action of Tregs is only starting to become understood. Proposed mechanisms for suppression of activated T cells, which are not completely elucidated yet, are cell contact-dependent suppression, limitation of growth factors and the production and secretion of inhibitory cytokines such as IL-10 and TGF-β[119].

5.3 Mast Cells

Another inflammatory cell type of the innate immune system, the mast cell[120,121], has been shown to accumulate in the rupture-prone shoulder region of human atheromas[122]. Additionally, activated mast cells at rupture sites of human coronary artery specimens were demonstrated to contain proteases such as tryptase and chymase[123-126]. Moreover, human coronary artery specimens were seen to contain TNF-α-rich activated mast cells[127,128], with the capacity to aggravate the ongoing inflammatory response and destabilize plaques[129]. Not only intimal inflammation but also inflammation of the arterial adventitia was shown to influence the plaque vulnerability[130]. Activated mast cells have been identified in the adventitia of vulnerable and ruptured lesions in patients with myocardial infarction[131-133] and more importantly, their number was found to correlate with the incidence of plaque rupture and erosion[131]. As mast cells are particularly abundant in the perivascular adventitia and near the neovessels of atherosclerotic lesions, and are regarded as a major source of a plethora of angiogenic and pro-inflammatory mediators and histamine[133], their activation will cause vascular leakage, chemotaxis to the atheroma and angiogenesis and apoptosis in the atheroma, all of which are adverse features in disease development (Figure 3). Recently, it has been demonstrated that systemic mast cell activation during athero-

genesis leads to increased plaque progression in ApoE$^{-/-}$ mice[134]. Moreover, focal activation of mast cells in the adventitia of advanced carotid artery plaques promotes macrophage apoptosis, microvascular leakage and *de novo* leukocyte influx, which culminates in a greatly enhanced incidence of intraplaque hemorrhage. Stabilization of the mast cells by cromolyn was seen to prevent these pathophysiological events. Sun *et al.* demonstrated that absence of mast cells, and in particular the mast cell-derived IL-6 and IFN-γ, decreased atherosclerotic lesion development in LDLr$^{-/-}$[135].

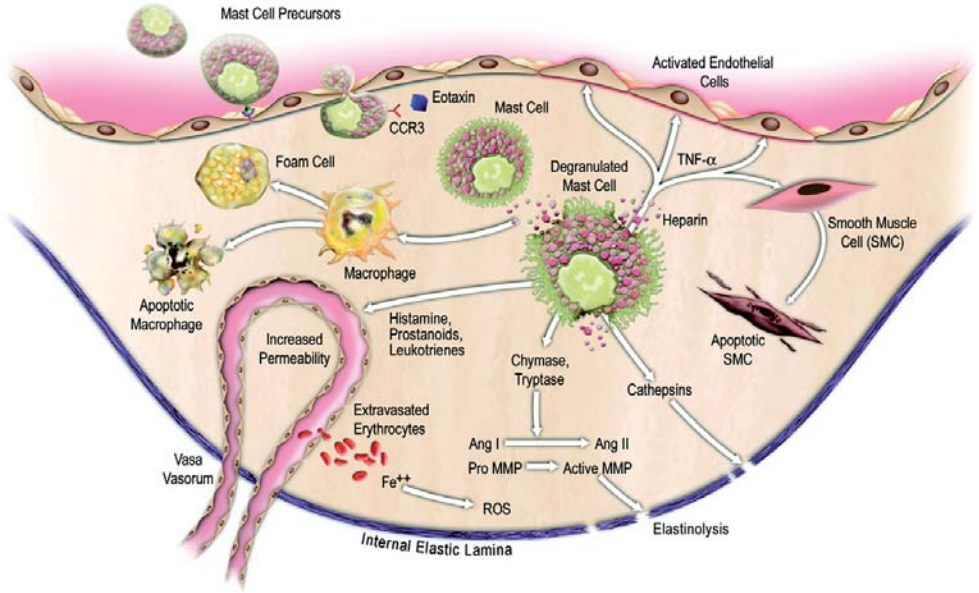

Figure 3. Putative functions of mast cells during atherogenesis. (Adapted from Libby P, Shi GP. *Circulation.* 2007;115:2471-2473)[136].

As mast cells contain, among others, a range of proteases (chymase, tryptase, cathepsins and MMPs), histamine, heparin, growth factors (vascular endothelial growth factor, basic FGF) and cytokines such as TNF-α, it is obvious that these cell types will have a strong impact on plaque stability. Mast cell proteases are capable of direct degradation of the extracellular matrix (ECM) components (e.g. collagen), necessary for plaque stability[137], but also indirectly via chymase/tryptase-induced activation of MMP-1 and -3[138,139]. Mast cell-derived IL-6 and IFN-γ can further destabilize plaque integrity by inducing cathepsin expression in endothelial cells and SMCs, promoting ECM degradation[135]. Furthermore, chymase induces SMC apoptosis by degrading fibronectin, a matrix component necessary for SMC adhesion and survival[140,141]. Activated mast cells are able to promote endothelial cell apoptosis mainly by secreting chymase and TNF-α[142]. Chymase secretion will lead to inactivation of focal adhesion kinase-mediated cell survival signaling, while TNF-α secretion will directly trigger apoptosis[143]. Recently, it has been demonstrated that activated subendothelial mast cells may have the capacity to affect endothelial erosion by releasing tryptase, chymase and cathepsin G, which are capable of degrading VE-cadherin and fibronection[144]. Furthermore, chymases convert Angiotensin I to pro-inflammatory Angioten-

sin II similar to angiotensin coverting enzyme (ACE), activate TGF-β and IL-1β and modulate lipid metabolism by degrading LDL, thus facilitating foam cell formation[145]. In conclusion, mast cells and derived granulae constituents can have profound effects on plaque morphology and stability, although it is not quite clear how and when mast cell are activated in atherosclerotic lesions and in its adventitia.

6. Vascular Smooth Muscle Cells

As previously described, VSMCs are one of the major cell types in atherosclerotic lesion development. Already at the early stages of lesion development growth factors and cytokines can induce phenotypic change of VSMCs from the quiescent "contractile" phenotype to the active "synthetic" phenotype, which can migrate from the media to the luminal side of the lesion where they accumulate subendothelially and proliferate. The migratory and proliferative activities of VSMCs are regulated by growth promoters such as PDGF, endothelin (ET)-1, thrombin, FGF, IL-1 and inhibitors such as heparin sulfates, nitric oxide (NO) and TGF-β. The MMPs could also participate in this process by catalyzing and removing the basement membrane and facilitating contacts of VSMCs with the interstitial matrix thereby promoting the change from quiescent, contractile VSMCs to cells capable of migration and proliferation. These responses are accompanied by accumulation of new ECM material, like collagen and proteoglycans, produced by VSMCs forming a fibrous cap covering the lipid core[24]. VSMCs are essential in the stability of atherosclerotic lesions and plaque rupture. Plaque rupture is often associated with an increase in fibrous cap macrophages and MMP production, while VSMC apoptosis is increased with concomitant reduced fibrous cap VSMC content. Therefore, factors that influence VSMC migration, proliferation and apoptosis are highly important in maintaining fibrous cap integrity[146]. For instance, the Th2 cytokine, IL-4 may decrease plaque stability by induction of SMC apoptosis and MMP-12 production[114]. Furthermore, mast cell-derived chymase induces VSMC apoptosis by degrading fibronectin, a matrix component necessary for VSMC adhesion and survival[140,141].
In addition, VSMCs are also very important in maintaining plaque stability as under inflamed conditions they can influence the ECM homeostasis. The VSMCs are largely responsible for controlling production versus breakdown of the ECM by production of collagen, elastin, glycoproteins and proteoglycans versus production of cathepsins and other extracellular matrix degrading enzymes such as MMPs[147] and ADAMs (A disintegrin and metalloproteinases)[148,149]. As previously mentioned IFN-γ can cause plaque destabilization at least in part by inhibiting VSMC proliferation and collagen production[32,33], but also by induction of cathepsin expression in SMCs thereby increasing extracellular matrix degradation[135]. These are only a few examples of how VSMCs can be influenced by a wide range of mediators from different cell types. Therefore, additional therapies to prevent fibrous cap thinning or matrix degradation are highly relevant to prevent clinical events in atherosclerosis.

7. Bioactive Lipids in Atherosclerosis

It has long been undisputed that dyslipidemia is instrumental in atherogenesis at all stages of disease progression. In addition to circulating lipids, intimal lipids were also regarded as prominent determinants of the biomechanical stability of the atherosclerotic plaques and are in fact used as an important criterion for plaque stability. The last decade of research has culminated in the recognition that lipids not only contribute to the disease as major constituents of the neointima, but also that specific lipids in the circulation as well as in plaques can independently modulate processes that are instrumental in disease initiation and progression. During atherogenesis, lipids accumulate in the core of the lesion. These lipids enter the plaque via influx of LDL, (β)VLDL and high-density lipoprotein (HDL) particles through vascular leakage and retention on proteoglycans[150]. These particles, mainly LDL, can become oxidized and are taken up by macrophages, rendering them foam cells. As these foam cells show unlimited uptake of lipids, they undergo apoptosis or necrosis, leading to the formation of a lipid core. Therefore, modified LDL (e.g. mmLDL, moxLDL or oxLDL) is widely recognized as a key factor in the pathogenesis of atherosclerosis and its thrombotic complications[151], as it activates endothelial cells, VSMCs and platelets, which are all involved in the progression of atherosclerosis. The modified LDL particles contain different atherogenic lipids, such as oxPAPC[152], lysophosphatidylcholine, phosphatidic acid, lysophosphatidic acid (LPA)[153,154] and sphingosine 1-phosphate (S1P). In this thesis, we shall focus on the two major bioactive phospholipids that were recently shown to be potentially important mediators in atherogenesis: LPA and S1P. While structurally unrelated, these lysolipids both act as agonists of G-protein-coupled receptor family members expressed on the surface of all vascular wall cell types involved in atherosclerosis, and are complementary in their mode of action. LPA showed to be an important mediator for the pro-thrombotic actions of LDL[153]. S1P, on the other hand, proved to be mainly associated with HDL, in which it contributed to its anti-atherogenic effects[155-157]. Chapter 2 will present the current knowledge on their homeostasis and their physiological activity in the context of atherosclerosis.

8. Study Aims

The bioactive lipids LPA and S1P appear to have opposing roles in atherogenesis. As LPA is considered pro-atherogenic, we aimed to outline LPA homeostasis during atherogenesis and study the effects of LPA on atherosclerosis development and stability. As S1P is considered anti-atherogenic, we aimed to delineate the role of S1P receptor agonism on atherogenesis.

9. Thesis Outline

The research described in this thesis is focused on two bioactive lysolipids, LPA and S1P. First, in chapter 2 a detailed review is given on the current status of LPA and S1P research and encompasses their formation and bioavailability, (possible)

involvement in atherosclerosis and cell type specific effects. In chapter 3 we established the suitability of the LDLr$^{-/-}$ atherosclerotic mouse model for evaluation of LPA homeostasis. In human atherosclerosis, LPA was found to accumulate in the lipid core of atherosclerotic lesions and identified as the primary platelet-activating lipid. Thus, it is conceivably, at least in part, that LPA is responsible for the thrombogenic activity of the plaque lipid core. The LPA content of advanced mouse lesions appeared to be very similar to that of human carotid artery specimens, and, in addition, we established in mouse lesions the accumulation of highly-unsaturated long-chain LPA species, which have high platelet-activating capacity. To further investigate the origin of lesion LPA, we performed expression profiling of key proteins in LPA metabolism and signaling. In chapter 4 we investigated localization of LPA species within the atherosclerotic lesions by imaging secondary ion mass spectrometry (SIMS). In this study we also tried to determine colocalization with other constituents of the lesions, and the lipid core in particular.

Chapter 5 and 6 describe studies in which LPA was administered either locally (chapter 5) or systemically (chapter 6). In human and mouse studies a relationship has been demonstrated between the mast cell content and activation status in the adventitia and severity of disease. LPA has recently been discovered to be a potent mast cell activator[158-160]. Therefore, local LPA challenge was performed on the adventitia of carotid artery lesions induced by perivascular collar placement in ApoE$^{-/-}$ mice to establish the effects of an LPA boost on mast cell activation and concomitant plaque destabilization. Besides effects on mast cells, LPA also has profound effects on other cell types such as endothelial cells, macrophages and VSMCs. To investigate long-term effects of increased plasma LPA concentrations on lesion development and morphology, we have induced carotid artery lesions in ApoE$^{-/-}$ mice that were treated systemically with LPA or phosphate buffered saline as a control by repeated intraperitoneal injection for 5 weeks.

In chapter 7 and 8 the effects of S1P receptor agonism on atherosclerosis development are illustrated. Numerous *in vitro* studies suggest that S1P, a bioactive lysosphingolipid associated with high-density lipoproteins, may account at least partly for the potent anti-inflammatory properties of HDL and, thereby, contributes to the anti-atherogenic potential attributed to high-density lipoproteins. We have investigated whether modulation of S1P signaling by FTY720, a sphingosine analogue, affects atherosclerosis in LDLr$^{-/-}$ mice (chapter 7). This sphingosine analogue can, in its phosphorylated form (FTY720-P), act on several S1P receptors. In addition, we have attempted to elucidate the underlying mechanism by which FTY720 can affect atherosclerosis. To further explore the effects of altering S1P signaling on atherogenesis, we performed a study on increased endogenous S1P availability by bone marrow transplantation with S1P lyase deficient bone marrow (chapter 8).

Finally, Chapter 9 provides a discussion of the most relevant findings of this thesis and an overview of future perspectives of these studies and their therapeutic implications.

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